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Clinical Assessment of *Lactobacillus gasseri* Strain, Selected by In-Vitro Tests

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Abstract: *Lactobacillus gasseri* G4 strain isolated from healthy human volunteer was selected as one of the best adhesive strain among a large group of lactobacilli. In-vitro tests for establishment of immunomodulatory potential of selected lactic acid bacteria were carried out. Three different analytical models were used for evaluation, according to the specifics of each different cytokine and to compare, where possible. The induction of cytokines like: interleukin-1 (IL-1), IL-10 and tumor necrosis factor alpha (TNF-α) were determined by widely used laboratory approach with mouse splenocytes as well as on combined (well-in-well) monocytic and epithelium human cell lines (THP-1 and HT-29). Other cytokines like IL-8 and IL-4 were evaluated by only one analytical model, while transforming growth factor beta (TGF-β) was evaluated by using all of the three models. The selected strain shows significant increase of IL-10, interferon gamma (IFN-γ) and TGF-β production in-vitro. Therefore, this strain was directed to clinical trials with 38 healthy volunteers, randomized and placebo-controlled. The results demonstrate statistically significant increase of TGF-β production (25%). Immunoglobulin A (IgA) and IFN-γ were also increased. Stable levels of IL-10 were observed after the in-vivo experiments with *L. gasseri* G4 strain. The selected strain does not increase in-vivo the pro-inflammatory cytokine IL-8. In this study the immunomodulatory properties of *L. gasseri* G4 were confirmed in-vivo therefore it is suitable for development of probiotic formulas.

Keywords: Probiotics, *Lactobacillus gasseri*, Immunomodulation, Cytokines, Immunoglobulin

1. Introduction

Human gastrointestinal tract is known to host trillions of microbes [1, 2]. Through co-evolution, the host not only tolerates but also evolved to necessitate the colonization by beneficial microbes, termed commensals, for multifaceted aspects of immune status and function [3]. One of the beneficial effects of probiotic strains is related with the modulation of the host's defenses which is most likely important for the prevention and treatment of infectious disease and also for treatment of intestinal inflammation. Probiotics may influence the immune system by means of products such as metabolites, cell wall components or DNA. In fact, these products can be recognized by the host cells sensitive for these because of the presence of a specific receptor [4]. The main target cells are generally the gut epithelial and the gut-associated immune cells. Finally, the interaction between probiotics and the host's immune cells by adhesion might be the triggering signaling cascade leading to immune modulation [5]. Lactic acid bacteria may enhance immune system function at the intestinal and systemic levels [6, 7, 8]. In humans, lactic acid bacteria have been shown to increase: B-lymphocytes or B cells, which recognize foreign matter [8], phagocytic activity, helping to destroy foreign matter [9], IgA-, IgG- and IgM-secreting cells and serum IgA levels, which would increase antibody activity [10] and γ-interferon levels, which help white blood cells fight disease [11]. As well, a large variety of immune cells are found in the gut mucosa. This allows the gut to interact with the immune system. Lactic acid bacteria can stimulate immune activity in the intestinal mucosa [12] and also probiotic consumption has been linked to nongastrointestinal disease prevention or

treatment such as shorter or less severe common cold episodes and may improve immunization [6]. A better understanding of the mutual interactions of the microbiota and host immune system would shed light on our endeavors of disease prevention and broaden the path to our discovery of immune intervention targets for disease treatment [13]. Cytokines are proteins which play an integrative role in the human immune response. The functions of these proteins are divers and include participation in normal T-cell-mediated immunity, the inflammatory response, cancer, autoimmunity and allergy [14]. Therefore, various pathologic conditions are accompanied by changes in cytokine levels. Proinflammatory cytokines like: TNF-α, IL-1, IL-6 and interferones are among the first cytokines produced in response to pathogenic bacteria [15]. In order to develop a better understanding of immune-mediated disorders, it becomes imperative to measure cytokine production [16]. The major immunoglobulin in serum is IgG (75%) and IgA is the second most common in serum. IgA is the major class of Ig in secretions - tears, saliva, colostrum, mucus. Since it is found in secretions IgA, called secretory, it is important in local (mucosal) immunity [17]. Studies clearly show that protection from infections of the upper respiratory tract is primarily due to local immunity, particularly immunoglobulin A (IgA) in mucosal secretions [18, 19, 20, 21].

In this study a lot of cytokines have been analyzed to conduct more comprehensive in-vitro/in-vivo evaluations. The usefulness of in-vivo studies is highlighted by demonstrations of Ig levels which cannot be evaluated in-vitro.

2. Materials and Methods

Bacterial strains- Lactobacillus gasseri G4 and the other three *Lactobacillus* strains were cultivated in MRS broth at 37°C. Two *Bifidobacterium* strains were cultivated in MRS broth with L-cysteine supplement at anaerobic conditions. The concentration was adjusted to 1.10^9 CFU/ml by centrifugation at stationary phase and resuspended in PBS buffer. All strains are isolated from healthy volunteers, men and women, at the age 20 to 40 years. All strains are possession of the LB Bulgaricum Plc collection.

Cell cultures and preparation:

Cell lines Caco-2 and HT-29 (ATCC) - were cultured in media consisting of DMEM (Invitrogen, USA) with 10% fetal bovine serum and in CO_2-incubator (5% CO_2) at 37°C.

Cell line THP-1 (ATCC) - was cultivated in media consisting of RPMI 1640 with 10% fetal bovine serum and in CO_2-incubator (5% CO_2) at 37°C.

Two component well-in-well cluster preparation: The two component cluster (Lab-Tek, Nunc Inc.) was prepared by using both HT-29 and THP-1 cell lines. Monolayers of HT-29 were cultivated in the upper wells, separated by membrane (0,45 μm). After fifteen days of maturing of HT-29, the second cell line THP-1 was cultivated in the lower well of the cluster. The cells were differentiated for 24 hours to macrophages in presence of 1 μg/ml phorbol-myristate-acetate (PMA). Than macrophages were washed with PBS (pH 7,4) to remove PMA and non-attached cells. To proceed, 200 μl suspension of the tested bacteria (concentration 1×10^9 CFU/ml) was added to 1,8 ml of RPMI media and then incubated in the lower well, together with the macrophages, and in the presence of the epithelium cell line (HT-29) in the upper well. After incubation for 20 hours in CO_2-incubator (5% CO_2) at 37°C supernatants were collected and centrifuged for measurement of cytokines.

Splenocytes isolation and preparation: Spleens from BALB/c mice, eight weeks old were used, cultivated in RPMI 1640 with 10% fetal bovine serum. The spleens were rubbed through sterile sieve (mesh - 100 μm), washed with RPMI 1640 and centrifuged at 135 g for 10 min. The pellets were resuspended in 5 ml 0,87% ammonium chloride for 2 min to remove erythrocytes. 100 μl of splenocyte suspension were transferred into each well and than 100 μl of bacterial suspension was added for evaluation after incubation in CO_2-incubator (5% CO_2) for 20 hours at 37°C. The supernatants were collected and centrifuged for measurement of cytokines.

Cytokines assay in-vitro was accomplished by the use of various enzyme-linked immunosorbent assay (ELISA) kits, according to the producer's instruction (Diaclone, USA). Murine ELISA kits were used for the supernatants obtained from splenocytes.

Human ELISA kits were used for the supernatants obtained from combined human epithelial and monocytic cell lines.

Each cytokine was evaluated in three separate analyses in the presence of control sample, containing only competent and/or epithelium cells and media, but no bacteria.

Clinical trial: Experiment was performed as randomized, placebo-controlled, open-label, prospective study of the in-vitro tested probiotic properties of a selected *Lactobacillus gasseri* strain. This study was carried out in diagnostic medical center, where 38 clinically healthy volunteers were previously selected according to a list of 16 criteria and demographic characteristics were recorded. Men and women, with average age of 39.2 years were randomly divided in two groups - one, consisting of 18 participants, was control group taking placebo and other, with 20 participants, was consuming the selected strain. All of them were also asked not to consume dietary supplements containing probiotics or prebiotics. All volunteers received their treatment pack of the product for the entire 30 - day course. Product was in freeze-dried sachet form for single daily intake *per os*, in dose of one table spoon after breakfast. Three visits were planed - two for taking blood samples and one for discussion with the volunteers about their condition at day- 15. Blood samples for determination of: cytokine, antibody (serum immunoglobulins) and inflammation blood marker- CRP were collected twice: at day- 0 and day- 30, before and after the intake of the preparation, containing (or not) the selected strain.

Blood parameters assay in-vivo: Blood samples were collected in 5 ml vacutainer tubes and subsequently

centrifuged at 1200 g/10 min with cooling. After separation the blood plasma was stored at -20°C until analyzed for the following: IFN-γ, IL-l0, IL-8, IL-12, IL-4, TGF-β and IgA, IgG with human ELISA kits (Diaclone, USA).

C - reactive protein (CRP) was evaluated by CRP Latex Test Kit - Serology test (Fisher HealthCare™ Sure-Vue™).

Statistical analyses: The analysis of data is made by descriptive statistical methods, using 95% confidence interval, showing number, average values, standard deviation, coefficient of asymmetry, dispersion and confidence interval for the relevant indicators.

For the evaluation of the measured, before and after intake of the preparation (or placebo), blood parameters is applied Student t-test for bidirectional testing of hypothesis with 95% confidence interval. In case of suspicion of deviation from the normal distribution of values, for some parameters, was administrated t-test with different dispersion between groups, or Wilcoxon signed-rank test (test for independent groups). As zero hypothesis was accepted the lack of difference (average differences = 0) between measurements before and after intake of the preparation (bidirectional test), as for statistically significant result was accepted value p< 0,05. The Excel®, Stata 14.1® software was used.

3. Results and Discussions

Lactobacillus gasseri G4 was selected due to its adhesion properties as previously reported by Dimitrov and Gotova, 2014 [22]. This strain also shows good technological properties (data not shown) for cultivation in milk and therefore it is suitable for further industrial use. The first stage of the present study was to evaluate in-vitro the immunomodulatory potential of the selected strain by measuring different cytokines. For this purpose each cytokine assay was performed by corresponding analytical laboratory approach. Table 1 contains the results of in-vitro tested cytokines: IL-10, IL-1, TNF-α, IL-8 evaluated on combined cell lines model (HT-29 and THP-1).

Table 1. *Cytokine concentrations obtained by using combined (epithelium/monocytic) cell lines (pg/ml).*

cytokine / strain	IL-10	IL-1	TNF-α	IL-8
L. gasseri G4	1860.6 ± 165.5*	107.8 ± 9.4	210.5 ± 14.0	271.6 ± 19.6
L. gasseri G7	1508.5 ± 115.0	146.3 ± 12.8	292.3 ± 16.2	52.9 ± 4.2
B. longum 3/15	1308.1 ± 114.8	101.1 ± 8.9	484.3 ± 21.1	58.7 ± 4.8
B. longum 10/48	1449.1 ± 102.6	140.3 ± 11.2	177.8 ± 12.3	46.9 ± 3.9
L. bulgaricus 58/1	1527.1 ± 112.3	107.1 ± 8.6	251.6 ± 19.5	78.1 ± 6.8
L. bulgaricus 130	531.6 ± 45.2	15.4 ± 1.2	134.3 ± 8.9	45.7 ± 3.9
control	4.8 ± 0.27	0.0 ± 0.01	0.8 ± 0.02	50.4 ± 3.7

Legend: * - mean value ± Standard Deviation (SD)

Epithelia cell lines produce basically IL-8 and TGF-β while other cytokines require immunocompetent cells like macrophages. However splenocytes comprise a variety of immune cell types with particular interactions, which makes them more representative, considering the complicated immune signaling in the body. TGF-β is released by both epithelial and immunocompetent cells. Therefore we found it appropriate to compare the results for some cytokines, evaluated on different laboratory models. As shown on Table 2, IL-10 and IL-1, TNF-α demonstrates higher but close values compared to the corresponding values in Table 1.

Table 2. *Cytokine concentrations obtained by using splenocytes (pg/ml).*

cytokine / strain	IL-10	IL-1	TNF-α	IL-4	IFN-γ
L. gasseri G4	1917.58 ± 182.2*	129.46 ± 9.8	244.69 ± 18.6	0.29 ± 0.02	3.91 ± 0.25
L. gasseri G7	1565.47 ± 148.0	168.02 ± 11.5	326.55 ± 28.0	0.61 ± 0.04	2.20 ± 0.17
B. longum 3/15	1365.08 ± 135.3	122.82 ± 9.6	518.50 ± 47.5	0.39 ±0.02	67.27 ± 4.90
B. longum 10/48	1506.07 ± 120.5	162.03 ± 11.5	212.03 ± 17.4	0.18 ± 0.02	8.32 ± 0.64
L. bulgaricus 58/1	1584.08 ± 122.5	128.80 ± 9.5	285.82 ± 19.6	1.46 ± 0.09	3.91 ± 0.22
L. bulgaricus 130	588.56 ± 40.2	37.07 ± 2.9	168.47 ± 14.8	0.32 ± 0.02	0.61 ± 0.04
control	6.8 ± 0.52	0.0 ± 0.01	0.9 ± 0.04	0.0 ± 0.01	0.0 ± 0.01

Legend: * - mean value ± SD

Table 3 presents the significant difference between TGF-β production on epithelial cell line Caco-2, combined cell lines (epithelium/monocytic) and splenocytes.

Table 3. *TGF-β concentrations analyzed by using different analytical models: epithelial cell line; combined (epithelium/monocytic) and splenocytes, pg/ml.*

cytokine / strain	TGF-β Caco-2	TGF-β HT-29/THP-1	TGF-β splenocytes
L.gasseri G4	72.6 ± 5.3*	259.0 ± 18.5	674.22 ± 48.5
L.gasseri G7	277.3 ± 20.2	279.3 ± 19.0	548.75 ± 41.0
B. longum 3/15	171.6 ± 13.9	347.1 ± 28.4	590.58 ± 45.6
B. longum 10/48	125.4 ± 9.1	306.4 ± 22.6	688.16 ± 48.9

cytokine / strain	TGF-β Caco-2	TGF-β HT-29/THP-1	TGF-β splenocytes
L.bulgaricus 58/1	257.5 ± 19.6	421.7 ± 31.5	716.04 ± 55.0
L.bulgaricus 130	59.4 ± 4.2	553.8 ± 37.6	890.29 ± 56.2
control	231.1 ± 14.2	343.7 ± 17.5	372.5 ± 18.0

Legend: * - mean value ± SD

Caco-2 synthesizes TGF-β even without being induced by bacteria, as shows the high level of the control. TGF-β values obtained from splenocytes are twice higher than on combined cell lines, which demonstrate the different power of these three analytical models. Other cytokines, like IL-4 and IFN-γ are detectable only on splenocytes. Selected cytokines were chosen to present cytotoxic (IFN-γ, IL-1, TNF-α); anti-inflammatory (IL-10, comparing to the pro-inflammatory IL-8; TGF-β) and humoral (IL-4) immunomodulatory effects. Due to our experience with hundreds of strains, IL-4 is rarely induced and not more than 5 pg/ml. According to its high induction of IL-10 and TGF-β and also with its relatively low induction of pro-inflammatory IL-8 and TNF-α, *L. gasseri* G4 shows moderate anti-inflammatory potential. Strains *L. gasseri* G7 and *Bifidobacterium longum* 10/48 (Table 1) demonstrate optimal ratio between high levels of IL-10 and low IL-8, which is appropriate for future clinical trials with patients suffering from inflammatory bowel diseases. IFN-γ values of *L. gasseri* G4 are relatively high among the *Lactobacillus* strains we have examined, but significantly lower comparing *B.longum* 3/15, as shown on Table 2. It is important to mark that the other strains from Tables: 1, 2 and 3 are chosen to represent higher, moderate or lower values in comparison to the selected strain *L. gasseri* G4.

As the main target of this study was to select a representative *L. gasseri* strain, according to its in-vitro evaluated high levels of IL-10, TGF-β and IFN-γ and in addition: high adhesive, good cholesterol-reduction properties (data not shown), industrial acceptable, and also its GRAS (generally recognized as safety) status, we found this *L. gasseri* G4 promising and proceeded to clinical trial with volunteers for evaluation of its potential in-vivo. All volunteers completed the study. Among the list of in-vivo tested cytokines, IL-4 and IL-12 show insignificant difference, as expected, in levels before and after 30 days intake of the freeze-dried strain and also between the control group and the representative one (data not shown). Almost the complete lack of IL-8 induction is expected due to the moderate in-vitro results and desirable in order to avoid pro-inflammatory effect (Table 4), which is also proved by the negative results of the inflammation blood marker C-Reactive Protein (< 6 mg/l is considered negative). Unlike the expectations on IL-10 levels, according to the in-vitro results, those from in-vivo tests were poor. A slight difference was noticed, basically in keeping the level among the representative group compared to the slight decrease among the control group (Table 4).

Table 4. *Concentration of IL-8 and IL-10 in both Representative and Placebo groups before and after intake (in blood plasma, pg/ml) ($p < 0.05$).*

Experimental groups	Type of Interleukin		
	n	IL-8	IL-10
Rep.group- before	20	0.39 ± 0.05*	2.52 ± 0.29
Rep.group - after	20	0.70 ± 0.09	2.53 ± 0.32
Placebo g.- before	18	0.86 ± 0.11	2.83 ± 0.34
Placebo g.- after	18	1.12 ± 0.19	2.68 ± 0.24

Legend: * - mean value ± SD

This excludes the strong anti-inflammatory effect suggested in-vitro. Correlation between in-vitro and in-vivo results is visible for IFN-γ. Values from representative and placebo group are close but when calculated, the increase of IFN-γ in the representative group is 26,7 %, compared to the slight increase in the placebo group of 10 %. This difference of almost 17 % real increase of the cytokine is now visible (Table 5).

Table 5. *Concentration of IFN-γ and TGF-β in both Representative and Placebo groups before and after intake (in blood plasma, pg/ml) ($p < 0.05$).*

Experimental groups	Type of Cytokines		
	n	IFN-γ	TGF-β
Rep.group- before	20	4.01 ± 0.39*	244.60 ± 21.5
Rep.group - after	20	5.08 ± 0.40	306.55 ± 23.0
Placebo g.- before	18	5.87 ± 0.48	256.72 ± 19.2
Placebo g.- after	18	6.49 ± 0.50	205.22 ± 16.5

Legend: * - mean value ± SD

As to the in-vivo obtained results for TGF-β (Table 5), it was completely confirmed that strain *L. gasseri* G4 is able to induce strongly this anti-inflammatory cytokine. New look on the entire immunological effect, from the intake of a certain bacterial strain over the macro organism, is revealed by the evaluation of immunoglobulin- A and G. The increase of IgA with over 20% among the representative group in comparison with the unchanged level of the same among the

placebo group (Table 6) is to prove the immunomodulatory properties of the selected strain.

Table 6. *Concentration of IgA and IgG in both Representative and Placebo groups before and after intake (in blood plasma, g/l) (p < 0.05).*

Experimental groups	Type of Immunoglobulin		
	n	IgA	IgG
Rep.group- before	20	2.10 ± 0.12*	10.53 ± 0.95
Rep.group - after	20	2.58 ± 0.15	10.54 ± 0.92
Placebo g.- before	18	2.29 ± 0.14	10.92 ± 1.10
Placebo g.- after	18	2.31 ± 0.15	10.89 ± 1.02

Legend: * - mean value ± SD

In addition, none of the volunteers had abnormal levels of IgA after the intake of the probiotic strain (Ref.: 0,7÷4,0 g/l) which strengthened the positive effect for the consumers' health. No differences were detected regarding changes of Ig G (Table 6) in both probiotic and placebo groups, before and after the intake of the strain/placebo preparation. The increase of IgA might be due to the fact that probiotics contact directly with mucosa associated lymphoid tissue, stimulating local (mucosal) immunity and humoral response.

4. Conclusions

The selected strain *Lactobacillus gasseri* G4 demonstrates immunomodulatory properties with in-vivo proven induction of TGF-β, IFN-γ and increasing the levels of IgA, without causing abnormal increase. It was not proved in-vivo an anti-inflammatory effect, unlike the expectations based on in-vitro results for Il-10, but it is proven as not pro-inflammatory strain due to Il-8 results. It was shown that probiotic supplementation may influence systemic antibody response, with strain specific effects. Being GRAS, strongly adhesive to epithelium and with its technological properties, this strain is suitable for future development of probiotic products.

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